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Original article

Phytochemical profiling of burrowing nematode (*Radopholus similis*) resistant and susceptible banana (*Musa* spp.) genotypes for detection of marker compounds

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Summary

Introduction – **Worldwide, the burrowing nematode, *Radopholus similis* is amongst most dreadful pests of banana. Host plant resistance is a powerful tool to tackle the nematode menace. Even though *R. similis* resistance and tolerance sources are abundant in the *Musa* gene pool, the progress on nematode resistance *Musa* breeding is not adequate. Hence understanding the mechanism of *R. similis* resistance and identification of markers becomes inevitable for successful banana breeding programmes.** *Materials and methods* – **Root extracts of two resistant ('Yankambi km-5' and 'Pisang Lilin') and two susceptible ('Robusta' and 'Nendran') *Musa* genotypes were prepared using water, methanol, ethyl acetate and n-hexane solvents. Effects of direct exposure of *R. similis* to water or solvent root extracts of resistant and susceptible genotypes were studied in bioassays *in vitro*. The phytochemical compound analysis of the solvent root extracts of resistant or susceptible genotypes was performed by gas chromatography-mass spectrometry (GC-MS).** *Results and discussion* – **Water extracts of both resistant and susceptible genotypes did not have any effect on *R. similis*. The solvent extracts of resistant genotypes inhibited the motility of *R. similis* when compared to those of susceptible genotypes. Phytochemical profiling of the tested *Musa* genotypes revealed that 29 methanol-soluble, 54 ethyl acetate-soluble and 24 n-hexane-soluble compounds were detected in the root extracts of banana. The number of compounds present among the genotypes varied. Three compounds such as 9-(2',2'-dimethylpropanoilhydrazono)-3,6-dichloro-2,7-bis-[2-(diethylamino)-ethoxy]fluorene from methanol extract, heptadecanoic acid, 9-methyl-, methyl ester from ethyl acetate extract and disulfide, 1-methylethyl propyl from n-hexane extract were commonly found in the two resistant genotypes, and were absent in the susceptible ones.** *Conclusion* – **The root extracts (methanol, ethyl acetate or n-hexane soluble) of resistant genotypes were inhibitory to *R. similis*. The three phytochemical compounds:9-(2',2'-dimethylpropanoilhydrazono)-3,6-dichloro-2,7-bis-[2-(diethyl amino)-ethoxy]fluorene;heptadecanoic acid, 9-methyl-, methyl ester; and disulfide, 1-methylethyl propyl can be considered as pre-infectional marker-like compounds for *Radopholus similis* resistance in banana.**

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Significance of this study

What is already known on this subject?

- Breeding in *Musa* genomes for the resistance or tolerance to burrowing nematodes *Radopholus similis* is a worldwide nonstop thrust.
- Post-infection chemical compounds responsible for *R. similis* resistance or tolerance are already known in *Musa* spp.

What are the new findings?

- The root extracts (methanol, ethyl acetate and n-hexane soluble) of resistant genotypes ('YKM-5' and 'Pisang Lilin') were biologically active against *R. similis*.
- Three pre-infection chemical metabolites were unique in the *R. similis* resistant genotypes and absent in the susceptible ones.

What is the expected impact on horticulture?

- The novel three pre-infection metabolites identified from the *R. similis* resistant genotypes can be considered as marker-like compounds and will be helpful to develop early or rapid screening protocols for detecting *R. similis* resistance in *Musa* spp.

Keywords
India, banana, *Musa* spp., pest control, plant breeding, resistance mechanism

Résumé

Profil phytochimique de génotypes de bananiers (*Musa* spp.) résistants ou sensibles au nématode foreur (*Radopholus similis*) pour l'identification de composés marqueurs.

Introduction – **Dans le monde entier, le nématode foreur du bananier, *Radopholus similis*, compte parmi les ravageurs les plus redoutables de cette culture. La résistance des plantes hôtes est un outil puissant de lutte contre la menace des nématodes. Même si les sources de résistance et de tolérance à *R. similis* sont abondantes dans le pool génétique des *Musa*, les progrès de la sélection des Musacées pour la résistance aux nématodes sont encore insatisfaisants. Aussi, la compréhension du mécanisme de résistance à *R. similis* et l'identification de marqueurs sont incontour-**

nables pour la réussite des programmes de sélection sur bananiers. *Matériel et méthodes* **– Des extraits racinaires de deux génotypes résistants ('Yankambi km-5' et 'Pisang Lilin') et de deux *Musa* sensibles ('Robusta' et 'Nendran') ont été préparés à partir d'eau ou de solvants tels que le méthanol, l'acétate d'éthyle et l'hexane. Les effets de l'exposition directe de *R. similis* à des extraits racinaires aqueux ou issus de solvants, de génotypes résistants et sensibles ont été étudiés dans des essais biologiques *in vitro*. L'analyse des composés phytochimiques des extraits racinaires de génotypes résistants ou sensibles a été effectuée par chromatographie en phase gazeuse couplée à la spectrométrie de masse (GC-MS).** *Résultats et discussion* **– Les extraits aqueux des génotypes résistants ou sensibles n'ont eu aucun effet sur *R. similis*. Les extraits au solvant des génotypes résistants ont inhibé la motilité de *R. similis* par rapport à ceux des génotypes sensibles. Le profil phytochimique des génotypes de *Musa* testés a révélé que 29 composés solubles au méthanol, 54 solubles à l'acétate d'éthyle et 24 à l'hexane ont été détectés dans les extraits de racines de bananier. Le nombre de composés présents parmi les génotypes a présenté des variations. Trois composés tels le 9-(2',2'-diméthylpropanoilhydrazono) -3,6-dichloro-2,7-bis- [2- (diéthylamino) -éthoxy] fluorène extrait au méthanol, l'acide heptadécanoïque, 9-méthyl-, ester méthylique extrait à l'acétate d'éthyle et le disulfure, 1-méthyléthyl propyle extrait à l'hexane se sont retrouvés communément chez les deux génotypes résistants, et étaient absents chez les génotypes sensibles.** *Conclusion* **– Les extraits racinaires (solubles au méthanol, à l'acétate d'éthyle ou à l'hexane) de génotypes résistants ont inhibé *R. similis*. Les trois composés phytochimiques: le 9-(2',2'-diméthylpropanoilhydrazono) -3,6-dichloro-2,7-bis-[2-(diéthylamino)-éthoxy] fluorène; l'acide heptadécanoïque, 9-méthyl-, méthyle ester; et le disulfure, 1-méthyléthyl propyle, peuvent être considérés comme des composés de type marqueurs de pré-infection pour la résistance à *Radopholus similis* chez le bananier.**

Mots-clés
Inde, bananier, *Musa* spp., lutte anti-ravageur, mécanismes de résistance, amélioration des plantes

Introduction

Banana (*Musa* spp., *Musaceae*), a global fruit and food crop, is cultivated in 150 countries across the tropical and subtropical regions of the world (Suryaprabha and Satheeshkumar, 2015). The production of banana is severely threatened by many pests and diseases, among which the burrowing nematode, *Radopholus similis* (Cobb, 1893) Thorne, a major pest responsible for destructive yield losses throughout the world (Seenivasan, 2017b). *Radopholus similis* is a migratory endo-parasite that moves freely inside the banana roots, feeds on root cortical cells and kills the cortical cells. Cell death results initially in reddish brown or dark brown lesions and later many death areas, rot or decay on the roots and corm. The dead or decayed roots fail to supply water and nutrient to the plant, what affects growth, development and finally reduces i) bunch weight; ii) harvested bunch number; and iii) duration of production cycles. It therefore directly affects bunch weight by reduced plant performance. In addition, the nematode-infested plants are in loss of plant anchorage with risk of toppling the whole plant during wet and windy weather, particularly at the bunch maturation stage. The estimated yield losses due to *R. similis* in banana range 15 to 80% per crop cycle (Sarah, 1989; Speijer and Kajumba, 2000; Seenivasan, 2017a). Injuries to the root facilitate entry of other soil borne pathogens such as *Fusarium oxysporum* f. sp. *cubense* (E.F.Sm) W.C. Snyder & H.N. Hansen which cause panama wilt disease (Selvaraj *et al.*, 2014).

Chemical nematicide is frequently used to control the nematodes in banana, what poses concerns of potential residues, ground water contamination, lethal effect on non-target organisms and field applicator safety (Niessen *et al.*, 2006). Though many bio-control agents including *Purpureocillum lilacinum* and *Glomus intraradices* proved effective in greenhouse conditions, their efficacy under field conditions has not been validated (Elsen *et al.*, 2008; Mendoza and Sikora, 2009). Under field conditions, other bio-control agents such as *Glomus mossae* and *Pseudomonas fluorescens* failed to protect plants in ratoon cycle (Seenivasan, 2017a). Cultural control measures such as corm-paring followed by hot water treatment and use of healthy suckers or clean tissue-cultured planting material offer only temporary control since fields are infested (Speijer *et al.*, 1999). Hence, the use of *R. similis* resistant cultivars or hybrids is a promising, economical and ecologically sound alternative for managing nematodes (Das *et al*., 2010).

Breeding programs in Trinidad, Jamaica, Honduras, Nigeria, Uganda, Brazil, France, Cameroon, Austria, Belgium, Taiwan, Spain, Tanzania and India are aiming to introduce commercially viable *Musa* cultivars or hybrids with *R. similis* resistance (Stover and Buddenhagen, 1986; Persley and George, 1996; Das *et al.*, 2014). The resistance or tolerance to *R. similis* in *Musa* spp. has been initially identified in the AAA genotype 'Yangambi km-5' ('YKM-5') and in the AA genotype 'Pisang Jari Buaya'. Resistance or tolerance has been later reported in the AAA genotypes 'Gros Michel', 'Mbwazirume', 'Marau', 'Nfuuka', 'Entukura', 'Tereza' and 'Kazirakwe'; AAB genotypes 'Kunnan' and 'Prata Enana'; ABB genotypes 'Karpooravalli', 'Pisang Awak', 'Saba' and 'Gia Hiu'; AA genotypes 'Paka', 'Calcutta-4', 'Pora Pora', 'Kopopo', 'Pisang Mas', 'Pisang Batuau', 'Anaikomban', 'Matti', 'Namarai' and 'Pisang Lilin'; and AB genotypes 'Padalaimoongil', 'Thenkunnan' and 'Sukali-ndizi' (Wehnut *et al*., 1978; Collingborn and Gowen, 1997; Speijer and Ssango, 1999; Viaene *et al.*, 2003; Dochez *et al*., 2006; Quénéhervé *et al.*, 2009; Seenivasan, 2017a). Some of the identified sources of resistance or tolerance are being now utilized for developing new hybrids resistant or tolerant to *R. similis* (Das *et al*., 2010).

The screening for *R. similis* resistance in *Musa* is mainly based on the observation of the reproduction factor (Cook and Starr, 2006), root lesion index, corm grade (Wehunt *et al*., 1978) and root morphology (Inamahoro *et al*., 2011). However, the methodology described in the literature for evaluating host plant responses to *R. similis* has not been fully validated (Viaene *et al.*, 2003), and an early or rapid screening methodology for detecting *R. similis* resistance through the marker technology is sought by many *Musa* breeders. Santos *et al.* (2009) characterized *Musa* accessions 'Borneo', 'Grand Naine', '1304-06', '4249-05', '0337-02', '0323-03' and '4279-06' using RAPD markers and reported the potential of RAPD markers for *R. similis* resistance. However, molecular markers are successful in the cases where monogenetic traits

or dominant genes predominate. In banana, this approach works only to a limited extent due to the polyploidy nature and polygenetic nematode resistant trait. Photochemical levels are more closely linked to phenotypes than to genotypes and as such can be used as predictive markers (Steinfath *et al.*, 2010).

Different types of phytochemical mechanisms were postulated for nematode resistance in banana. Higher levels of preformed phenolic cells in 'YKM-5' and higher numbers of lignified cell walls in vascular bundles in 'Pisang Jari Buaya' were reported as instrumental for resistance (Fogain and Gowen, 1995). Valette *et al.* (1998) suggested that the presence of constitutive lignin, dopamine, flavonoids, caffeic and ferulic acids might act as a barrier for entry and colonization of *R. similis* in roots of 'YKM-5'. Luis (1998) claimed a group of plant secondary metabolites called phenylphenalenones as *R. similis* resistant compounds in *Musa* as it was produced in larger quantities in resistant cultivars. Collingborn *et al.* (2000) found that the condensed content of tannins consisting of procyanidin and propelargonidin units was higher in 'Kunnan', 'Dwarf Cavendish' and 'YKM-5'. Further histochemical analysis studies also observed the presence of constitutive lignin (Wuyts *et al.*, 2007) and phenolic compounds (Dhakshinamoorthy *et al*., 2014) in *R. similis* resistant plants and reported them as involved in resistance mechanisms. Hölscher *et al.* (2014) identified greater concentrations of phenylphenalenone-related compounds in a resistant in comparison with a susceptible *Musa* cultivar. The same authors showed some of these compounds as nematostatic and nematocidal, causing *R. similis*-immobility rates from 54 to 89%. Although phenolic and lignin compounds are known to be formed in nematode resistant banana roots in response to infection by *R. similis*, the key pre-infectional phytochemical compounds responsible for resistance have not yet been studied. The phytochemical profile of *Musa* roots is needed to identify compounds related to *R. similis* resistance. Biologically active compounds in plants are soluble in different solvents. In this study *R. similis* was exposed to root extracts of resistant and susceptible *Musa* genotypes under *in vitro* conditions. The phytochemical compounds in root extracts of resistant versus susceptible genotypes were profiled and compared using gas chromatography coupled to mass spectrometry (GC-MS).

Materials and methods

Plant materials

The *R. similis* resistant cultivars Yankambi km-5 ('YKM-5') and 'Pisang Lilin' and the susceptible cvs. Robusta and Nendran were obtained from the Banana Gene Bank, Department of Fruit Crops, Horticultural College and Research Institute, Tamil Nadu Agricultural University, Coimbatore, India. The field site was maintained with nematicide treatments to ensure nematode-free planting materials. Five equal-sized corms from each accession were collected from mother plants. The corms were paired with a knife and soaked in hot water at 55 °C for 15 min. The corms were planted in pots (30 × 20 × 18 cm) containing sterilized pot mixture (red loam, sand and farm yard manure in a 2:1:1 ratio v/v/v) and placed in a glass house at 28 ± 4 °C. The plants were irrigated once per day and fertilized with 20-20-20 (N-P-K) fertilizer at a 0.1% concentration at 20-day intervals. Ninety days after planting, the pseudostem was cut slightly above soil level from each pot and corm with roots were carefully de-potted. Corms with roots were washed in tap water and wiped with filter paper. The roots were cut from the corm base, pooled by genotype and stored at -5 °C. The test root material was confirmed nematode-free by the centrifugal floatation technique (Barker and Niblack, 1990).

Nematode testing

The population of *R. similis* used in these studies was originally isolated from the banana cv. Grand Naine roots from the Orchard, Horticultural College and Research Institute, Tamil Nadu Agricultural University, Coimbatore, India. The nematodes were extracted from the infested roots, using the centrifugal floatation technique (Barker and Niblack, 1990). Mixed life stages of *R. similis* (J2, J3, J4 and adults) were recovered and multiplied on carrot disc cultures (Pinochet *et al*., 1995). The mixed life stages of *R. similis* populations were obtained by macerating the carrot disks in a blender followed by sieving (Speijer and De Waele, 1997).

Extraction procedure of phytochemicals

A 250-g root sample of each banana genotype was used to prepare water, methanol, ethyl acetate and n-hexane extracts. The 250-g roots were mixed with 500 mL distilled water, milled using a warring blender for 1 min. The water homogenate was then centrifuged at 10,000 rpm for 10 min and supernatant was lyophilized at -10 °C using a lyophilizer (SENTRY, The Virtis Company Inc., NY, USA). The resulting freeze-dried water extract powders were stored in -20 °C and used for bioassay. The residue remains in the centrifuge were collected in a 1-L flask and soaked in 500 mL methanol for 48 h. Then it was centrifuged at 10,000 rpm for 10 min. The supernatant was collected and evaporated on a rotary evaporator at 50 °C. The methanol extract was collected from evaporator and stored in -20 °C and used for bioassay. The residue was then extracted sequentially with ethyl acetate followed by n-hexane to get ethyl acetate and methanol extracts and stored in -20 °C and used for bioassay.

Bioassay

The bio-efficacy of water, n-hexane, methanol and ethyl acetate extracts of the four *Musa* genotypes was tested against *R. similis.* Assays were carried out in 96-well plates. Each extract was dissolved in 50% dimethyl sulfoxide (DMSO) to obtain concentrations of 500 µg mL^{-1}. Mixed life stages of *R. similis* were diluted in distilled water to 20 nematodes 100 μL^{-1} aliquot. A 180-µL aliquot of the nematode suspension was delivered to each well of a 96-well plate followed by 20 µL of test extracts comprising 6 replicate wells for each genotype treatment. The control wells were filled with 180 µL nematode suspension and 20 µL of 50% DMSO. The plate was covered with Parafilm® and placed in a humid chamber. Motility of *R. similis* was assessed by viewing plates with an inverted compound microscope at a magnification of 40× after 24, 48 and 72 h. Inhibiting effects were confirmed by transferring *R. similis* to distilled water after 72 h in the test suspensions. The nematodes were considered to be immobile if they did not move after probing with a fine needle and did not resume motility after 48 h in distilled water. The bioassays of water, n-hexane, methanol or ethyl acetate extracts were repeated once. The corrected percent immobility was calculated using the formula:

$$\text{Corrected immobility (\%)} = \frac{T - C}{100 - C} \times 100$$

where T is the percent immobility in treatment and C is the

percent immobility in control. An extract of a genotype was considered active if it caused more than 10% immobility compared to the solvent control.

Phytochemical profiling

The methanol, ethyl acetate and n-hexane extracts were re-dissolved in 1 mL methanol, ethyl acetate or n-hexane for the subsequent gas chromatography coupled to mass spectrometry analysis (GC-MS) in order to identify the compounds from methanol, ethyl acetate and n-hexane extracts using a Shimadzu GC-MS – QP2010 plus (Figures 1–3). The gas chromatograph was equipped with a DB-1 capillary column (30 m × 0.25 mm × 0.25 µm). Helium was used as gas carrier at flow rate of 0.86 mL min^{-1}. The column temperature program was set at 80 °C for 3 min isotherm, increased to 250 °C at a rate of 3 °C min^{-1} and held for 1 min. A mass spectrometer with a scan range of masses from 40–500 *m/z* was used**.** The ionization was set at 70 eV. The injector, a 10-n source and the transfer line temperature were set at 250, 250 and 275 °C, respectively. The individual compounds were identified by comparison of their mass spectra with those from NIST and Wiley library mass spectral database.

Statistical analysis

The data were analyzed using SPSS 16.0 for Windows software (SPSS Inc., Chicago, IL, USA). The percentage immobility data was tested for normal distribution using Shapiro-Wilk test and they did not follow normality. However, the arc sin transformed data were found to fit a normal distribution in Shapiro-Wilk test. Hence data were arc sin transformed before subjected to ANOVA. Means were separated by Duncan's multiple range test (Gomez and Gomez, 1984). The transformed data were back transformed for presentation.

Results and discussion

***In vitro* bio-assay**

The percent immobility of *R. similis* when exposed to water extracts of different banana genotypes ranged from 0.0 to 1.7% (Table 1). Methanol extracts of genotypes caused 7.1–33.1% immobility at 24 h, 7.1–33.1% at 48 h and 7.5–33.7% at 72 h. Immobility was high (31.7–33.1% at 24 h, 31.7–33.5% at 48 h and 32.0–33.7% at 72 h) on resistant genotypes where as low (7.1–8.7% at 24 h, 7.1–9.0% at 42 h and 7.5–9.0% at 72 h) in susceptible genotypes. In the ethyl acetate extract bioassay, 'Pisang Lilin' caused significantly higher immobility (38.1% at 24 h, 38.3% at 48 and 72 h) than in the solvent control ($P < 0.05$), followed by 'YKM-5' (21.0% at 24 h, 21.5% at 48 h and 21.7% at 72 h), whereas the two susceptible genotypes caused significantly lower immobility (5.5–6.7% at 24 h, 5.7–7.0% at 48 h and 5.7–7.3% at 72 h). The n-hexane extracts of genotypes also showed significant variation on immobility of *R. similis*. Immobility was significantly high (32.4–36.3% at 24 h, 32.7–36.5% at 48 h and 33.0–36.7% at 72 h) in the resistant genotypes where as low (7.6–9.1% at 24 h, 7.9–9.3% at 48 h and 8.3–9.3% at 72 h) in the susceptible genotypes (Table 1).

The water extracts of all the tested genotypes during the *in vitro* bioassays did not have any inhibitory effect on *R. similis* whereas methanol, ethyl acetate and n-hexane extracts caused immobility of *R. similis* in the resistant genotypes. The active compounds responsible for nematode resistance are not water soluble in nature. Many studies evidenced that the

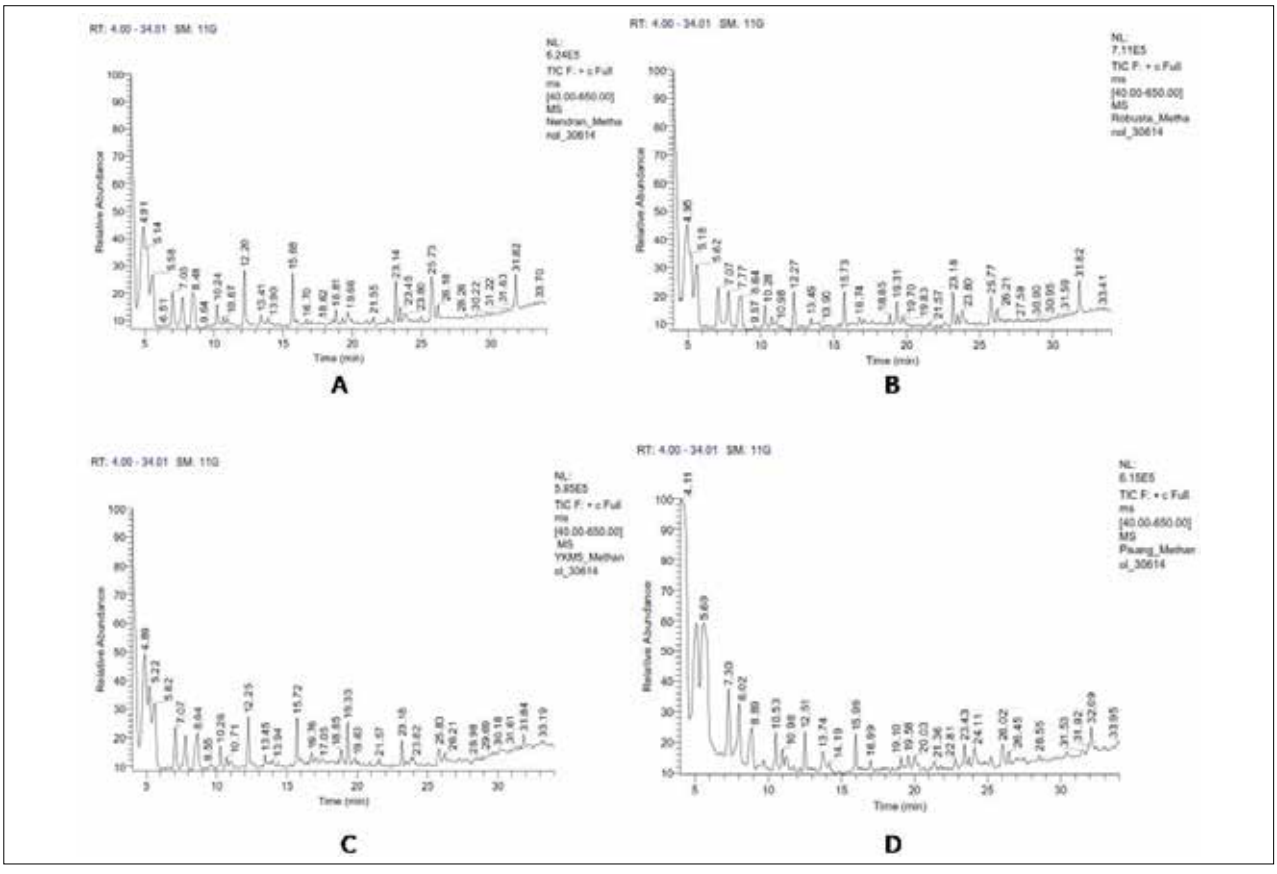

Figure 1. GC-MS chromatograms of methanol root extracts of *Musa* genotypes A) 'Nendran', B) 'Robusta', C) 'YKM-5', and D) 'Pisang Lilin'.

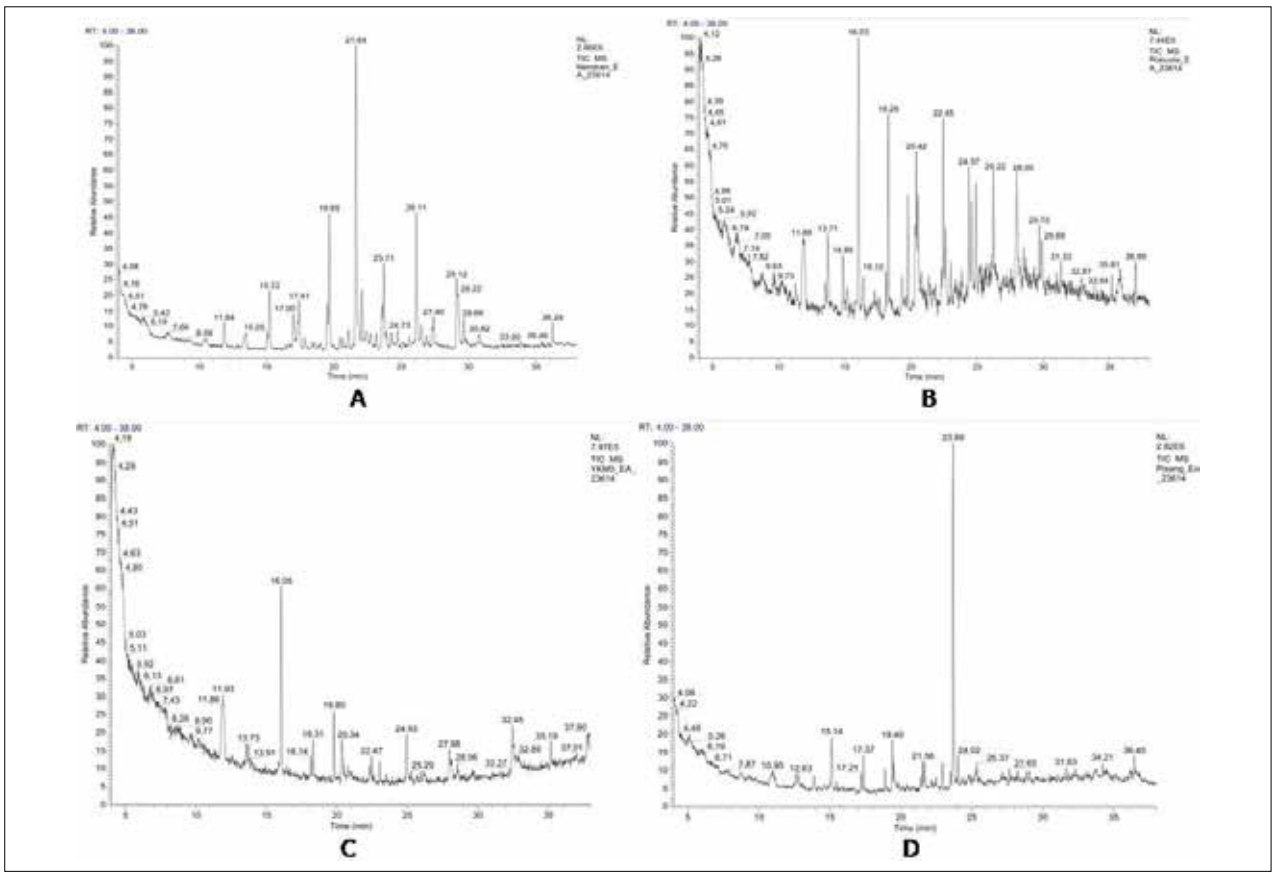

FIGURE 2. GC-MS chromatograms of ethyl acetate root extracts of *Musa* genotypes A) 'Nendran', B) 'Robusta', C) 'YKM-5', and D) 'Pisang Lilin'.

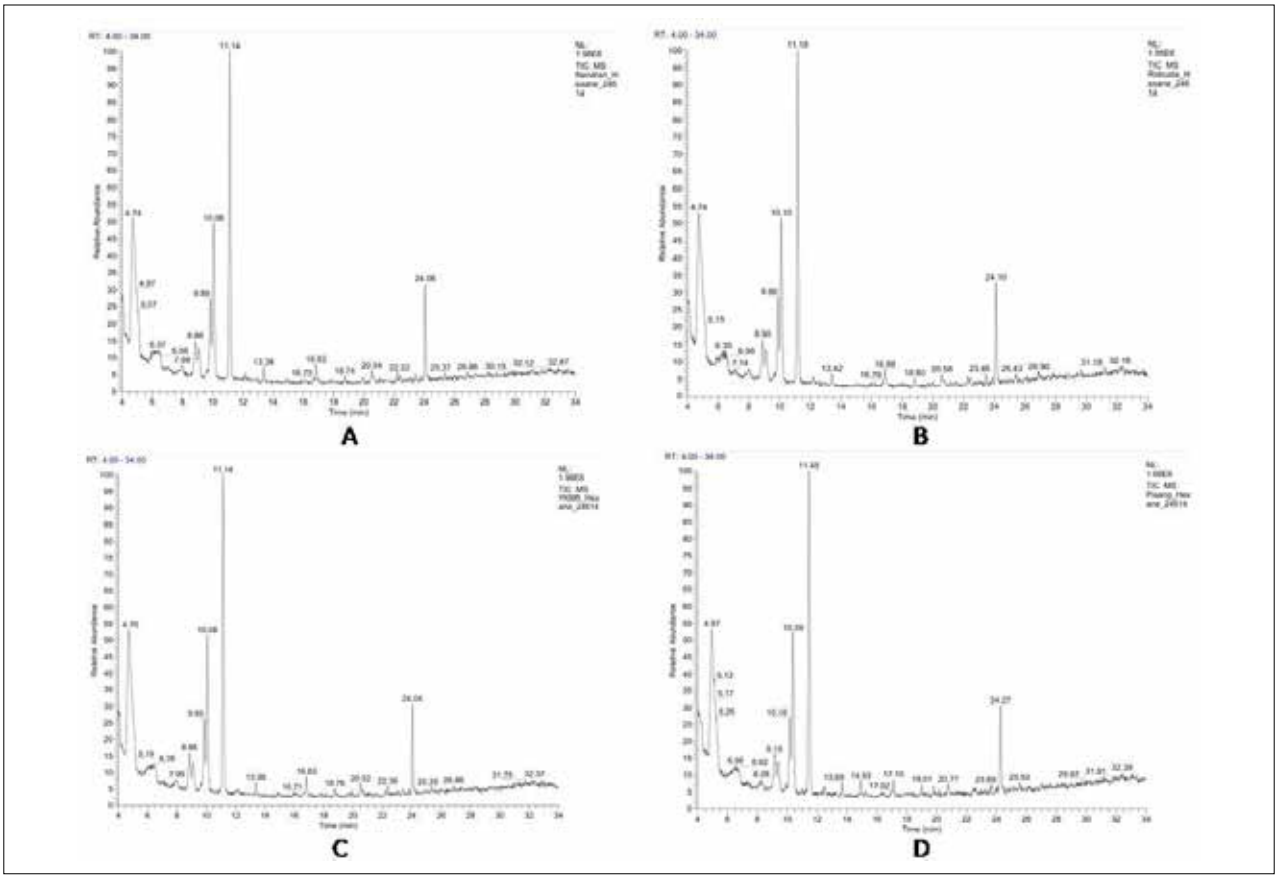

FIGURE 3. GC-MS chromatograms of n-hexane root extracts of *Musa* genotypes A) 'Nendran', B) 'Robusta', C) 'YKM-5', and D) 'Pisang Lilin'.

Table 1. Percent immobility of *Radopholus similis* when exposed to water, methanol, ethyl acetate and n-hexane extracts of *Musa* genotypes *in vitro* condition. The values are means (n = 10) of immobility observed at 24 h, 48 h and 72 h after exposure.

Musa genotypes	Water			Methanol			Ethyl acetate			n-Hexane		
	24 h	48 h	72 h	24 h	48 h	72 h	24 h	48 h	72 h	24 h	48 h	72 h
'YKM-5'	1.7 a	1.7 a	2.0 a	33.1 a	33.5 a	33.7 a	21.0 b	21.5 b	21.7 b	36.3 a	36.5 a	36.7 a
'Pisang Lilin'	1.5 a	1.7 a	1.7 a	31.7 a	31.7 a	32.0 a	38.1 a	38.3 a	38.3 a	32.4 a	32.7 a	33.0 a
'Robusta'	1.3 a	1.3 a	1.5 a	7.1 b	7.1 b	7.5 b	5.5 c	5.7 c	5.7 c	9.1 b	9.3 b	9.3 b
'Nendran'	0.3 a	0.3 a	0.3 a	8.7 b	9.0 b	9.0 b	6.7 c	7.0 c	7.3 c	7.6 b	7.9 b	8.3 b

Means in a column followed by the same letter are not significantly different at $P < 0.05$ according to Duncan's multiple range test.

solubility of bioactive compounds can be improved by suitable solvents when compared to water (Rauha *et al*., 2000; Kagale *et al.*, 2004). In this study, methanol, ethyl acetate and n-hexane proved to be good solvents to extract bioactive compounds from the roots of *Musa* genotypes. The methanol, ethyl acetate and n-hexane extracts of resistant *Musa* genotypes showed a strong inhibitory effect on *R. similis* whereas the susceptible genotypes had very weak activity. The resistant genotypes might contain specific compounds to cause immobility. It is also possible that more than one compound is involved in the resistance to *R. similis* as methanol, ethyl acetate and n-hexane soluble extracts showed inhibitory effects. Veech (1979) first observed that the root extracts of resistant cotton plants inhibited the movement of *M. incognita*. Hölscher *et al.* (2014) reported that phenylphenalenone-related compounds from *R. similis* induced necrotic lesions in a resistant and susceptible *Musa* cultivar. The critical concentration of active phenylphenalenones was low in susceptible *Musa* roots and high in resistant *Musa* roots. This study established the pre-infectional nematostatic effect of the roots of *Musa* resistant genotypes.

Phytochemical profiling

A total of 29 compounds were detected in the methanol-fraction, 54 metabolites in the ethyl acetate fraction and 24 compounds in the n-hexane-fraction from the root extracts of the banana genotypes (Tables 2–4). However, the number of compounds present in each genotype varied. 'YKM-5' roots had 27 compounds and 'Pisang Lilin' had 36 compounds. Root extracts of 'Nendran' and 'Robusta' had 51 and 40 compounds respectively (Tables 2–4). The number of metabolites identified from root extracts (methanol, ethyl acetate and n-hexane soluble) of four tested genotypes was not uniform. The type of compounds among the genotypes also varied. Similar variation of metabolites among potato cultivars, resistant or susceptible to late blight disease caused by *Phytophthora infestans* (Mont.) de Bary, and among apple cultivars resistant and susceptible to aphids, *Myzus persicae* (Sulzer, 1776), supports our observations in banana (Laothawornkitkul *et al.*, 2010; Wang *et al.*, 2014).

Methanol root extracts

The methanol root extracts all contained oxime-, methoxy-phenyl-compounds (Figure 1, Table 2). The benzenepropanoic acid, 3,5-bis(1,1-dimethylethyl)-4-hydroxy-, methyl ester was present in both susceptible genotypes, with a high peak in 'Pisang Lilin'. The compound 9-(2',2'-dimethylpropanoilhydrazono)-3,6-dichloro-2,7-bis-[2-(diethyl amino)-ethoxy]fluorine was found in both the resistant genotypes and not in the susceptible ones. The compounds 4-trifluoroacetoxypentadecane and methyl 3-cis,9-cis,12-cis-octadecatrienoate were found only in 'YKM-5'. Arsenous acid, tris(trimethylsilyl) ester; d-glycero-d-ido-heptose; 1h-indole-2,3-dione, 5-chloro-1-(trimethylsilyl)-; stearic acid, 3-(octadecyloxy)propyl ester; (5á)pregnane-3,20á-diol, 14à,18à-[4-methyl-3-oxo-(1-oxa-4-azabutane-1,4-diyl)]-, diacetate; trans-13-octadecenoic acid; cyclopropanebutanoic acid, 2-[[2-[[2-[(2- pentylcyclopropyl)methyl]cyclopropyl] methyl]cycloprop; 1-monolinoleoylglycerol trimethylsilyl ether; and [1,1'-bicyclopropyl]-2-octanoic acid, 2'-hexyl-, methyl ester were all found only in 'Pisang Lilin'.

The compound 9-(2',2'-dimethylpropanoilhydrazono)-3,6-dichloro-2,7-bis-[2-(diethylamino)-ethoxy]fluorine might have a strong role in *R. similis* resistance in *Musa* spp. as it was found in the resistant genotypes and not in the susceptible ones (Table 2). This compound is produced by *Streptomyces parvulus* and proved to have antimicrobial, antioxidant and cytotoxic effect (Naine *et al.*, 2015). It has been isolated from medicinal and aromatic plants such as *Madhuca nerifolia* (Moon) H.J. Lamand, or *Coriandrum sativum* L. (Sukumaran and Mathew, 2013; Asif *et al*., 2014). Cell death and necrosis, accumulation of toxins and modification of cell walls have been ascribed as resistance mechanisms against nematodes in plants (Atkinson *et al.*, 2003). Hence the cytotoxic principle of the compound 9-(2',2'-dimethylpropanoilhydrazono)-3,6-dichloro-2,7-bis-[2-(diethyl amino)-ethoxy] fluorine may be involved in *R. similis* resistance mechanism by sealing off the infected tissues to limit nematode spreading. However, MS imaging technique studies are needed to confirm the hypothesis of the "sealing effect" of these compounds in the necrotic lesions.

The resistant genotypes had several specific compounds: 4-trifluoroacetoxypentadecane and methyl 3-cis,9-cis,12-cis-octadecatrienoate were found only in 'YKM-5'. The 4-trifluoroacetoxypentadecane was reported to have antimicrobial, antibacterial and antiviral activity (El-Baz *et al*., 2015). Arsenous acid, tris(trimethylsilyl) ester was found only in 'Pisang Lilin', and was reported to have antioxidant and antimicrobial activities in addition to nematostatic effects (Barathikannan *et al.*, 2016). The d-glycero-d-ido-heptose is a sugar moity compound involved in antimicrobial activity (Appelt *et al.*, 2013). Stearic acid, 3-(octadecyloxy) propyl ester is derivative of fatty acid that has a carboxyl functional group. The 1-monolinoleoylglycerol trimethylsilyl ether is an alkaloid compound that has antimicrobial and antioxidant activities (Sheela and Uthayakumari, 2013). The [1,1'-Bicyclopropyl]-2-octanoic acid, 2'-hexyl-, methyl ester has some insecticide property (Gnanavel and Saral, 2013). Most of the compounds found only in the resistant genotypes are biologically active.

Ethyl acetate root extracts

The compound Hexadecanoic acid, methyl ester was commonly found in all the tested genotypes. However, the peak size was high for susceptible genotypes and medium for resistant genotypes (Figure 2, Table 3). Heptadecanoic

acid, 9-methyl-, methyl ester was found only in the resistant genotypes. The compounds 3-trifluoroacetoxydodecane; quinoline, 1,2-dihydro-2,2,4-trimethyl-; 12,15-octadecadiynoic acid, methyl ester; benzene, (1-ethylundecyl)-; benzene, (1-methyldodecyl)-; spirost-8-en-11-one, 3-hydroxy-, (3á,5à,14á,20á,22á,25R)-; 9,12,15-octadecatrienoic acid, 2,3-bis[(trimethylsilyl)oxy]propyl ester, (Z,Z,Z)- were found only in the cv. Pisang Lilin. The compounds pentadecane, 7-methyl-; pentadecane, 7-methyl-; 2-propenoic acid, tridecyl ester; methyl 9-cis,11-trans-octadecadienoate; 9-octadecenamide, (Z)- and 9-(2',2'-dimethylpropanoilhydrazono)-3,6-dichloro-2,7-bis-[2-(diethyl amino)-ethoxy]fluorene were found only in the cv. YKM-5.

The compound hexadecanoic acid, methyl ester has been previously reported in the banana fruit (Meechaona *et al*., 2007), and our results confirm that it is also a common chemical constituent in roots of *Musa* species. Since it was upregulated in the two susceptible genotypes, it is suggested that this compound is involved in plant-nematode interactions. A compound found only in the resistant genotypes is the heptadecanoic acid, 9-methyl-, methyl ester which possesses antioxidant and antimicrobial properties according to Lalitharani *et al.* (2010). The nematicidal effects of methyl or ethyl esters of several fatty acids, including heptadecanoic acid, speculate that it might directly involve *R. similis* resistance by biosynthesis of nematode inhibiting compounds when roots are infected with *R. similis* (Kisenwether *et al.*, 2014).

The compounds 3-trifluoroacetoxydodecane;quinoline, 1,2-dihydro-2,2,4-trimethyl-; 12,15-octadecadiynoic acid, methyl ester; benzene, (1-ethylundecyl)-; benzene, (1-methyldodecyl)-; spirost-8-en-11-one, 3-hydroxy-, (3á,5à,14á,20á,22á,25R); and 9,12,15-octadecatrienoic acid, 2,3-bis[(trimethylsilyl)oxy]propyl ester, (Z,Z,Z)- are unique for 'Pisang Lilin'. Among them, trifluoroacetoxydodecane is known to have antimicrobial and anticancer activities (Sudha *et al.*, 2013); quinoline, 1,2-dihydro-2,2,4-trimethyl- to have antioxidant property and toxic principles (Sitarek and

Table 2. Phytochemical profile and retention time of methanol root extracts of four *Musa* genotypes.

Identified compounds	'Nendran'	'Robusta'	'YKM-5'	'Pisan Lilin'
Oxime-, methoxy-phenyl-	++ (4.23)	++ (4.25)	++ (4.25)	+++ (4.37)
Arsenous acid, tris (trimethylsilyl) ester				+++ (7.30)
N-Methyladrenaline, tri-TMS	++ (13.41)		+++ 13.45)	
d-Glycero-d-ido-heptose				+ (9.70)
2H-1,4-Benzodiazepin-2-one	+ (10.67)			
Tetraacetyl-d-xylonic nitrile	+ (10.96)			++ (13.76)
1H-Indole-2,3-dione, 5-chloro-1-(trimethylsilyl)-				++ (10.98)
Stearic acid, 3-(octadecyloxy)propyl ester				++ (11.23)
(5á)Pregnane-3,20á-diol, 14à,18à-[4-methyl-3-oxo-(1-oxa-4-azabutane-1,4-diyl)]-, diacetate				+ (14.19)
Mono-TMS of (pyridoxine-H_2O)		++ (16.74)		
Pterin-6-carboxylic acid		++ (19.70)	++ (16.76)	
4-Trifluoroacetoxypentadecane			++ (19.33)	
2-Propenoic acid, tridecyl ester		++ (19.33)		
trans-13-Octadecenoic acid				++ (19.58)
Imidazole, 2-amino-5-[(2-carboxy)vinyl]-	++ (19.66)			
Pentadecanoic acid, 14-methyl-, methyl ester	++ (23.14)		++ (23.18)	
Hexadecanoic acid, methyl ester		++ (23.16)		
Cyclopropanebutanoic acid, 2-[[2-[[2-[(2- pentylcyclopropyl)-methyl] cyclopropyl]methyl]cycloprop				++ (23.41)
Benzenepropanoic acid, 3,5-bis(1,1-dimethylethyl)-4-hydroxy-, methyl ester	++ (23.45)	++ (23.49)		
Phthalic acid, butyl undecyl ester	++ (23.80)			
1-Monolinoleoylglycerol trimethylsilyl ether				++ (25.25)
Methyl 10-trans,12-cis-octadecadienoate	+++ (25.73)			
8,11-Octadecadienoic acid, methyl ester		++ (25.77)		
Methyl 3-cis,9-cis,12-cis-octadecatrienoate			++ (25.83)	
[1,1'-Bicyclopropyl]-2-octanoic acid, 2'-hexyl-, methyl ester				++ (26.02)
Heptadecanoic acid, 9-methyl-, methyl ester	++ (26.18)	++ (26.21)	++ (26.21)	
Diisooctyl phthalate	++ (31.82)			
Phthalic acid, di(2-propylpentyl) ester		++ (31.82)		
9-(2',2'-Dimethylpropanoilhydrazono)-3,6-dichloro-2,7-bis-[2-(diethyl amino)-ethoxy]fluorine			++ (31.84)	++ (31.92)

+ : Compounds detected and size of the peak is low; ++: Compounds detected and size of the peak is medium; +++: Compounds detected and size of the peak is high; Figures in brackets are retention times (Rt) in min.

Table 3. Phytochemical profile and retention time of ethyl acetate root extracts of four *Musa* genotypes.

Identified compounds	'Nendran'	'Robusta'	'YKM-5'	'Pisang Lilin'
sec-Butyl nitrite	++ (10.50)			
Decane, 4-methyl-		++ (11.28)	++ (13.75)	
1-Butoxy-1-isobutoxy-butane	+++ (11.84)			
3-Trifluoroacetoxydodecane				++ (12.61)
Undecane, 2,8-dimethyl-		+++ (13.73)		
Quinoline, 1,2-dihydro-2,2,4-trimethyl-				++ (13.89)
4H-Pyrrolo[3,2,1-ij]quinoline, 1,2,5,6-tetrahydro-4-methyl-		++ (14.87)		
Octadecane, 6-methyl-	++ (19.01)	++ (15.18)		
Tetradecane	+++ (15.22)			
Phenol, 2,4-bis(1,1-dimethylethyl)-	++ (17.83)	++ (16.40)		
3-Acetoxydodecane				
1-Undecanol	+++ (17.00)			
1-Hexadecanol, 2-methyl-	++ (24.73)	++ (17.23)		
Hexadecane	+++ (19.65)	+++ (18.28		+++ (17.37)
Pentadecane, 7-methyl-			++ (18.31)	
Hexadecane, 1-chloro-	++ (18.39)			
Benzeneacetic acid, 4-tetradecyl ester	++ (18.57)			
Dodecyl acrylate	++++ (21.64)			+++ (19.40)
2-Propenoic acid, tridecyl ester			++ (20.36)	
Heptadecane, 2,6,10,14-tetramethyl-		+++ (20.40)	++ (22.45)	
Benzene, (1-butylheptyl)-	++ (20.44)			
Benzene, (1-propyloctyl)-	++ (20.67)			
10-Octadecenal		++ (20.75)		
Tetradecane, 2,6,10-trimethyl-		+++ (24.37)		+++ (21.56)
Heptadecane, 2,6-dimethyl-				
Octadecanal	++ (22.08)			
9,12,15-Octadecatrienoic acid,	++ (24.25)			++ (22.16)
Benzene, (1-pentyloctyl)-	++ (22.43)			
Octadecane	+++ (23.71)	+++ (22.45)		
12,15-Octadecadiynoic acid, methyl ester				++ (22.49)
1-Monolinoleoylglycerol trimethylsilyl ether		++ (35.79)		++ (28.23)
Benzene, (1-ethylundecyl)-				++ (22.95)
1-Chloroeicosane	++ (23.15)			
Benzene, (1-methyldodecyl)-				++++ (23.69)
Ethyl iso-allocholate		++ (23.84)		++ (24.81)
Hexadecanoic acid, methyl ester	+++ (26.14)	+++ (24.91)	++ (24.95)	++ (24.02)
2-Myristynoyl pantetheine		++ (25.20)		
Octadecane, 3-ethyl-5-(2-ethylbutyl)-	+++ (27.40)	+++ (26.24)		++ ((25.35)
Heneicosane, 11-(1-ethylpropyl)-	++ (25.62)			
Heptadecane, 9-hexyl-				
Benzenepropanoic acid, 3,5-bis(1,1-dimethylethyl)-4-hydroxy-methyl ester	+++ (26.49)			
Phthalic acid, butyl undecyl ester	++ (26.88)			
Heptadecanoic acid, 9-methyl-, methyl ester			++ (28.54)	++ (27.67)
Methyl 9-cis,11-trans-octadecadienoate			++ (28.00)	
Heptadecanoic acid, 16-methyl-, methyl ester	+++ (29.66)			
9,12-Octadecadienoic acid, methyl ester, (E,E)-	+++ (29.14)			
Ethanol, 2-(octadecyloxy)-	++ (30.77)			
Spirost-8-en-11-one, 3-hydroxy-, (3á,5à,14á,20á,22á,25R)-				++ (32.29)
9-Octadecenamide, (Z)-			+++ (32.47)	
9,12,15-Octadecatrienoic acid, 2,3-bis[(trimethylsilyl) oxy]propyl ester, (Z,Z,Z)-				++ 34.50)
9-(2',2'-Dimethylpropanoilhydrazono)-3,6-dichloro-2,7-bis-[2-(diethyl amino)-ethoxy]fluorine			++ (35.21)	
Diisooctyl phthalate	++ (36.24)			
7,11Dioxapentacyclo[15.3.0.0(4,16).0(5,13).0(5,10)]eicos-13-en-20-ol-8-one, 1á,12,12-trimethyl-		++ (36.99)		

+ : Compounds detected and size of the peak is low; ++: Compounds detected and size of the peak is medium; +++: Compounds detected and size of the peak is high; ++++: Compounds detected and size of the peak is very high; Figures in brackets are retention times (Rt) in min.

Table 4. Phytochemical profile and retention time of n-hexane root extracts of four *Musa* genotypes.

Identified compounds	'Nendran'	'Robusta'	'YKM-5'	'Pisang Lilin'
1,2-Benzisothiazol-3-amine tbdms		++ (4.10)		++ (4.26)
p-Xylene	++++ (4.74)	++++ (4.76)	++++ (4.72)	++++ (4.97)
Propane, 1,1'-sulfonylbis-		++ (8.01)	++ (7.97)	++ (8.26)
Disulfide, 1-methylethyl propy			+++ (8.86)	+++ (9.17)
Disulfide, dipropyl	+++ (8.86)	+++ (8.90)		
n-Propyl sec-butyl disulfide	+++ (10.06)	+++ (10.10)	+++ (10.08)	+++ 10.39)
Disulfide, bis(1-methylpropyl)	++++ (11.14)	++++ (11.18)	++++ (11.14)	++++ (11.47)
Oxalic acid, allyl heptyl ester	++ (12.15)			
sec-Butyl nitrite		++ (12.19)		
Heptadecane, 2,6,10,14-tetramethyl-	++ (18.76)			
Octadecane, 6-methyl-		++ (20.58)	++ (18.76)	
Tetradecane, 2,6,10-trimethyl-		++ (18.80)		
Heneicosane, 11-(1-ethylpropyl)-	++ (20.54)			
1-Hexadecanol, 2-methyl-				++ (20.75)
Dodecane, 5,8-diethyl-	++ (22.22)	++ (22.26)		
3-Benzoyl-2-t-butyl-4-isopropyl-4-methyl-oxazolidin-5-one	++ (22.39)			
Pentadecane, 2-phenyl-		++ (22.45)		
Benzene, (1-ethylundecyl)-		++ (23.48)	++ (23.42)	
Pentacosane, 13-phenyl-	++ (23.42)			
Benzoic acid, 4-methyl-, [4-(methoxycarbonyl)phenyl]methyl ester				++ (23.65)
Benzene, (1-methyldodecyl)-	+++ (24.06)	+++ (24.10)	+++ (24.04)	
Octadecane, 3-ethyl-5-(2-ethylbutyl)-	+ (28.23)		++ (25.37)	
2-Myristynoyl pantetheine		++ (25.41)		
Heptadecane, 9-hexyl-			++ (26.86)	

+ : Compounds detected and size of the peak is low; ++: Compounds detected and size of the peak is medium; +++: Compounds detected and size of the peak is high; ++++: Compounds detected and size of the peak is very high; Figures in brackets are retention times (Rt) in min.

Sapota, 2003); and 12,15-octadecadiynoic acid, methyl ester to have an antibacterial activity (Idan *et al.*, 2015). The 9,12,15-octadecatrienoic acid, 2,3-bis[(trimethylsilyl)oxy] propyl ester, (Z,Z,Z)- has been identified as one of the components responsible for resistance against *Fusarium oxysporum* f. sp. *albedinis*, causal agent of the Bayoud disease on date palm (Gaceb-Terrak and Rahmania, 2013).

The compounds uniquely present in 'YKM-5' are pentadecane, 7-methyl- and 2-propenoic acid, tridecyl ester which were reported to have antimicrobial activity (Rasekhi *et al*., 2014; Kumar *et al*., 2015); methyl 9-cis,11-trans-octadecadienoate was reported to have cytotoxic effect (Muthusamy *et al.*, 2015); and 9-octadecenamide, (Z)-was found to have antibacterial activity (Hadi *et al.*, 2016).

n-Hexane root extracts

Disulfide, 1-methylethyl propyl was observed in the two resistant genotypes but not in the susceptible ones (Figure 3, Table 4). It is a volatile sulphur compound reported to have nematicidal action (Tada *et al.*, 1988). Heptadecane, 9-hexyl- was only present in the resistant genotype 'YKM-5'. It was reported to have antifungal property (Abubacker and Palaniyappan, 2015). The compounds 1-hexadecanol, 2-methyl- and benzoic acid, 4-methyl-, [4-(methoxycarbonyl)phenyl] methyl ester were only present in the resistant genotype 'Pisang Lilin'. The 1-hexadecanol, 2-methyl- was reported to have antioxidant and antimicrobial activities (Hussein *et al*., 2015).

Conclusion

The root extracts (methanol, ethyl acetate and n-hexane soluble) of the two *Radopholus similis* resistant genotypes ('YKM-5' and 'Pisang Lilin') were inhibitory to *R. similis*. The comparison of the phytochemical profiles of resistant and susceptible genotypes revealed that the compounds 9-(2',2'-dimethylpropanoilhydrazono)-3,6-dichloro-2,7-bis-[2-(diethyl amino)-ethoxy]fluorene from methanol root extract; heptadecanoic acid, 9-methyl-, methyl ester from ethyl acetate root extract; and disulfide, 1-methylethyl propyl from n-hexane root extract, have all the potential of pre-infectional marker-like compounds for resistance to *R. similis* as they were found only in the resistant genotypes. Further studies on i) purification and isolation of these compounds from other resistant genotypes, ii) their reaction on *R. similis*, and iii) generation of simple assays to detect these compounds would validate them as resistance markers.

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